51. (Once Amended) The staphylokinase derivative of claim 47 labeled SY19 (S3C-MP5), wherein said derivative comprises amino acid substitutions S3C-MP5, E65D, K74R, E80A, D82A, K130T, K135R and wherein the cysteine at position 3 is chemically modified with MAL-PEG 5 kDa.

Please add new claims 61 and 62 as follows:

61. The staphylokinase derivative of claim 39, wherein the at least one amino acid substituted with Cys is at least one of a surface exposed residue, a charged residue, a threonine residue and a serine residue.

62. The staphylokinase derivative of claim 39, wherein the at least one amino acid substituted with Cys is the position of the polyethylene glycol coupling.

REMARKS

Claims 31-60 are pending in the application. Claims 31-38, 42, 44, 48-50 and 52-60 have been withdrawn from consideration. Claims 39-41, 43, 45-47 and 51 have been amended. New claims 61 and 62 have been added. Reexamination and allowance of the claims are requested.

The Examiner has objected to the Abstract of the Disclosure because of its length as it exceeds the 150-word limit pursuant to MPEP § 608.01(b). The Abstract of the Disclosure has been revised to meet the word limit requirement.

A clean copy of the claims, as requested by the Examiner, is found in Attachment A.

Support for new claim 61 is found in Fig. 1 of the specification. Support for new claim 62 is found on p. 5, lines 31-34 of the specification.

The Examiner has acknowledged the election of Group CXXXIV, containing claims 39-41, 43, 45-47 and 51, drawn to a staphylokinase derivative having the code SY-19 (S3C-MP5) as the specie for examination. The Examiner has requested that the elected claims,

particularly claim 43, be amended so that they do not encompass staphylokinase derivatives not elected. Claim 39, from which claim 43 depends, has been amended to limit the staphylokinase derivatives encompassed.

The Examiner has objected to the specification for spelling anomalies, for the absence of sequence identifiers, and for not complying with the preferred layout format. The spelling of "cysteine" has been corrected throughout the specification and claims, and the specification has been placed in the preferred arrangement. The Examiner has also objected to the abstract of the disclosure because of its length. The Abstract has been amended to meet the length limitation.

The Examiner has objected to the labeling of Figs. 1, 3 and 4, the line quality of Figs. 2A – 5B, and the legibility of the numbers, letters and reference characters of Figs. 1- 5B. New, corrected drawings have been submitted in response to the objections.

The Examiner has objected to the claims for containing references to amino acids by both 3-letter codes and full names. The claims have been amended to refer to amino acids by 3-letter codes only.

The Examiner also objects to claim 39 for failing to number limitations, claim 40 for the recitation of SakSTAR without reciting, in the first occurrence, the entire phrase for which the abbreviation is used, claim 46 for failing to clearly indicate the characteristics of the claimed derivative, and claim 51 for failing to clearly indicate the modifications of the staphylokinase derivative claimed. Amendments have been made to the claims according to the Examiner's suggestions, and to indicate that SakSTAR is a specific wild-type variant of staphylokinase.

The Examiner has rejected claims 38-41, 43, and 45-47 (and claim 51 dependent thereon) under 35 U.S.C. § 112, second paragraph, for indefiniteness. The Examiner states that claims 39, 40, 41 and 43 require a numerical sequence identifier. The claims have been amended to supply this identifier. The Examiner asserts that claim 39 is indefinite in the recitation of

"reducing reactivity with a panel of murine monoclonal antibodies." The claim has been amended to state that the antibodies are specific to staphylokinase. The Examiner asserts that claim 39 is indefinite in the recitation of "at least one amino acid substituted with Cys" and "polyethylene glycol substitution" because it is not expected that all of the claimed substitutions will result in the claimed properties. The claim has been amended to recite the substitutions that will produce the claimed properties. The Examiner asserts that claim 41 is indefinite in the recitation of "without reducing the specific activity by more than 50 percent." The claim has been amended according to the Examiner's suggestion to state that the specific activity of the wild type staphylokinase is the basis for the comparison. The Examiner asserts that claim 43 is indefinite in the recitation of "in which the indicated amino acids have been replaced by other amino acids." The Examiner maintains that it is unclear whether the claim is drawn to the derivatives listed in Tables 1, 3-8, 13, 19 and 20, or to variants of the listed derivatives. The phrase "in which the indicated amino acids have been replaced by other amino acids" has been removed to indicate that the claim is drawn to the derivatives listed in the tables.

The Examiner asserts that claims 45 and 46 are indefinite in the recitation of "chemically modified with polyethylene glycol with molecular weights up to 20 KDa. The molecular weight is that of the polyethylene glycol, not that of a staphylokinase derivative. Accordingly, claim 45 has been amended to read "chemically modified with polyethylene glycol, wherein the polyethylene glycol can have a molecular weight of up to 20 KDa." The Examiner asserts that claim 46 is indefinite in the recitation of "amino acids in the NH_2-terminal region of 10 amino acids are substituted with Cys." The claim has been amended, according to the Examiner's suggestion, to include a numerical sequence identifier and to indicate the positions of the amino acids numerically. The Examiner also asserts that claim 47 is indefinite in the recitation of "wherein the serine in position 2 or 3 is substituted with a cystein." The claim has

been amended, according to the Examiner's suggestion, to include a numerical sequence identifier.

The Examiner has rejected claims 39, 40 and 41 (and claims 45 and 46 dependent therefrom) under 35 U.S.C. § 112, for inadequate description. The Examiner asserts that no disclosure has been provided indicating a relationship between structural elements of staphylokinase that can be modified and the maintenance of 50% or more of the specific activity of the corresponding wild-type staphylokinase. The Examiner concludes that many functionally unrelated polypeptides are encompassed within the scope of the claims. According to claim 39 as amended, amino acid substitution with Cys is restricted to positions outside both the binding epitope and activation epitope of the staphylokinase molecule. The positions related to the binding epitope and the activation epitope are known to those in the art, and the form of their recitation in claim 39 represents a convenient way to refer to individual substitutions enabled by the specification. Similarly, polyethylene glycol substitution is limited to positions outside both the binding epitope and activation epitope of the staphylokinase molecule. Claim 39 is further limited to derivatives wherein the substitution allows the formation of a homodimeric form of staphylokinase through the formation of an intermolecular disulfide bridge. These limitations provide sufficient guidance so that undue experimentation would not be required to determine which staphylokinase derivatives would have at least 50% of the specific activity of the corresponding wild-type staphylokinase. For these reasons, the rejection of claims 39-41 and 45-46 for inadequate description is believed to have been overcome.

The Examiner has rejected claims 39, 40 and 41 (and claims 45 and 46 dependent therefrom) under 35 U.S.C. § 112, for lack of enablement. The Examiner asserts that, while the specification discloses the structure and function of the polypeptide of Figure 1 and two structural elements known to affect the activity of the staphylokinase, no disclosure of the critical structural elements required in any derivative of the species of Fig. 1 to retain at least 50% of the specific

activity of the corresponding wild-type staphylokinase has been presented. The Examiner concludes that undue experimentation would be needed by one skilled in the art to screen and isolate the molecules encompassed by the claim having the desired activity. According to claim 39 as amended, amino acid substitution with Cys is restricted to positions 1-10, and polyethylene glycol substitution is limited to the Cys components of the claimed derivatives. These limitations provide sufficient guidance so that undue experimentation would not be required to determine which staphylokinase derivatives would have at least 50% of the specific activity of the corresponding wild-type staphylokinase. For these reasons, the rejection of claims 39-41 and 45-46 for lack of enablement is believed to have been overcome.

The Examiner has rejected claims 39-41 under 35 U.S.C. § 102(e) for anticipation by U.S. Patent No. 5,801,037 to Behnke et al. (hereinafter "Behnke"). The Examiner asserts that Behnke teaches a staphylokinase variant of the species of Fig. 1 wherein a methionine residue at position 26 has been substituted with a cysteine residue. The Examiner concludes that because claims 39-41 are directed in part to staphylokinase derivatives of the species of Fig. 1 wherein one or more amino acids have been replaced by a cysteine residue, the staphylokinase of Behnke anticipates the claims of the present invention. However, claim 39 states that the substitution of the at least one amino acid with Cys results in dimerization and/or increased specific activity and/or reduced clearance and/or increased thrombolytic potency. There is no indication that the compound disclosed by Behnke possesses any of these properties. Furthermore, Behnke does not anticipate the derivatives of claim 39 because substitution of the at least one amino acid with Cys according to claim 39 is limited to positions outside the binding epitope and activation epitope. Behnke describes the substitution of Met at position 26 by Cys. However, Met 26 is within the binding domain of staphylokinase. For these reasons, the rejection of claims 39-41 over Behnke is believed to have been overcome.

The Examiner asserts that the subject matters of applications Serial No. 09/728,760 and 09/020,018 appears to be overlapping with the subject matter of the present application. Applicants will respond to a double patenting rejection, when and if raised, in light of the claims pending at that time.

In view of the above amendments and remarks, it is believed that the claims are in condition for allowance. Reconsideration of the rejections is requested. Allowance of claims 39-41, 43, 45-47, 51, 61 and 62 is respectfully requested.

Respectfully submitted,

WEBB ZIESENHEIM LOGSDON
ORKIN & HANSON, P.C.

By 

Thomas J. Clinton
Registration Number 40,561
Attorney for Applicant
700 Koppers Building
436 Seventh Avenue
Pittsburgh, PA 15219-1818
Telephone: (412) 471-8815
Facsimile: (412) 471-4094

MARKED-UP VERSION OF THE SPECIFICATION

On page 6, please delete and replace the current version of the first full paragraph starting at line 4 with the following replacement paragraph:

More in particular the serine in position 2 or 3 is substituted with a [cystein] cysteine and the [cystein] cysteine is chemically modified with polyethylene glycol having a molecular weight of 5, 10 or 20 kDa. Preferred embodiments of these derivatives are SY161(S3C-MP5), SY161(S3C-P10), Sy161(S3C-P20), SY19(S3C-MP5), SY19(S3C-SP5), SY19(S2C-SP5, S3C-SP5), SY19(S3C-P20), SY19(S3C-P10) all as defined in table 20.

On page 6, please delete and replace the current version of the second full paragraph starting at line 12 with the following replacement paragraph:

The presence of [cysteins] cysteines allows the formation of dimers of two staphylokinase derivatives of the invention.

On page 8, please delete and replace the current version of the first full paragraph starting at line 1 with the following replacement paragraph:

Fig 1. Protein sequence of wild-type staphylokinase, SakSTAR. Numbering starts with the NH2-terminal amino acid of mature full length staphylokinase (SEQ ID NO: 1).

On page 8, please delete and replace the current version of the third full paragraph starting at line 13 with the following replacement paragraph:

Fig 3. Protein sequence of wild-type staphylokinase, SakSTAR (SEQ ID NO: 1) with indicated amino acid substitutions.

squares: single amino acid substitutions;

circles: combined (2 to 3) amino acid to Ala substitutions.

On pages 12 and 13, please delete and replace the current version of the first full paragraph starting at page 12, line 25 and bridging page 14 and ending at line 25 with the following replacement paragraph:

The plasmids encoding SakSTAR(K35A,E38A,K74A,E75A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(K74A) and SakSTAR(E75A) were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24), using Vent DNA polymerase (New England Biolabs, Leusden, The Netherlands), and available or generated sakSTAR variants as template. Two fragments were amplified by PCR, the first one starting from the 5' end of the staphylokinase gene with primer 5'-CAGGAAACAGAATTCAGGAG-3' (SEQ ID NO: 2) to the region to be mutagenized (forward primer), the second one from the same region (backward primer) to the 3' end of the staphylokinase gene with primer 5'-CAAAACAGCCAAGCTTCATTCATTCAGC-3' (SEQ ID NO: 3). The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a new primerless PCR using Taq polymerase (Boehringer Mannheim). After 7 cycles (1 min at 94°C, 1 min at 70°C), the extended product was reamplified by adding the 5' and 3' end primers (see above) to the PCR reaction and by cycling 25 times (1 min at 94°C, 1 min 55°C, 1 min at 72°C). The final product was purified, digested with EcoRI and HindIII and cloned into the corresponding sites of pMEX602sakB. The plasmid encoding SakSTAR(E38A,K74A,E75A,R77A) was assembled by digestion of pMEX602sakB and pMEX.SakSTAR(K35A,E38A,K74A,E75A,R77A) with BpmI which cuts between the codons for K35 and E38 of SakSTAR, and ligation of the required fragments. The plasmid encoding SakSTAR(K35A,K74A,E75A,R77A) was constructed by digestion of

pMEX.SakSTAR(K35A,E38A,K74A,E75A,R77A) and pMEX.SakSTAR(K74A,E75A,R77A) with BpmI and religation of the required fragments. The plasmids encoding SakSTAR(K35A,E38A,E75A,R77A) and SakSTAR(K35A,E38A,K74A,R77A) were constructed by two PCR using pMEX.SakSTAR(K35A,E38A,K74A,E75A,R77A) as template, followed by restriction ligation and recloning into pMEX602sakB.

On pages 23 and 24, please delete and replace the current version of the second full paragraph starting at page 23, line 37 and bridging page 24 and ending at line 30 with the following replacement paragraph:

The variants SakSTAR(Y17A,F18A), SakSTAR(F104A), SakSTAR(F111A), SakSTAR(Y9A), SakSTAR(Y91A), SakSTAR(Y92A), SakSTAR(I87A), SakSTAR(I106A) and SakSTAR(I120A) were constructed with the Chameleon Double-Stranded Site-Directed Mutagenesis kit from Stratagene (La Jolla, USA), using the pMEX.SakSTAR vector as template, and following instructions of the supplier. The mutagenic oligonucleotides (not shown) were used in combination with the selection-primer LY34 5' CAAAACAGCCGAGCTTCATTCATTCAGC (SEQ ID NO: 4), which destroys the unique HindIII site located 3' to the staphylokinase encoding gene in pMEX.SakSTAR and allows to counter-select the non-mutant progeny by HindIII digestion. The deletion of the HindIII site was in most cases correlated with the presence of the desired mutation introduced by the mutagenic oligonucleotide. The variant SakSTAR(I133A), was constructed by performing a polymerase chain reaction on the pMEX.SakSTAR plasmid using the primer 818A located at the 5' end of the sakSTAR gene (5' CAGGAAACAGAATTCAGGAG) (SEQ ID NO: 2) and the mutagenic primer LY58 (5' TTCAGCATGCTGCAGTTATTTCTTTTCTGCAACAACCTTGG) (SEQ ID NO: 6). The amplified product (30 cycles: 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C) was purified, digested with EcoRI and PstI, and ligated into the corresponding sites of

pMEXSakSTAR. The variants SakSTAR(I128A), SakSTAR(L127A) and SakSTAR(N126V) were constructed by performing a polymerase chain reaction using the primer 818A located at the 5' end of the SakSTAR gene and mutagenic primers (not shown). The amplified product (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C) was purified, digested with EcoRI and StyI, and ligated into the corresponding sites of pMEXSakSTAR.

On page 25, please delete and replace the current version of the first full paragraph starting at line 5 with the following replacement paragraph:

The plasmids encoding all the other variants listed in Table 3 were constructed by direct PCR or by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using pMEX.SakSTAR or available plasmids encoding SakSTAR variants as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C, the first one starting from the 5' end (primer 818A) of the staphylokinase gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of pMEX.SakSTAR. For each construction, the sequence of the variant was confirmed by sequencing the entire SakSTAR coding region.

On pages 29 and 30, please delete and replace the current version of the second full paragraph starting at page 29, line 18 and bridging page 30 and ending at line 6 with the following replacement paragraph:

In an effort to maximize the activity/antigenicity ratio, these amino acids were substituted with other amino acids than Ala. As summarized in Table 5, substitution of K35 with [A, E or Q] Ala, Glu or Gln revealed that SakSTAR (K35A) had the most interesting properties, substitution of Y73 with [F, H, L, S or W] Phe, His, Lev, Ser or Trp did not rescue the marked reduction in specific activity, and K74 confirmed its key role in binding of antibodies from immunized patient plasma, the best specific activity/antigenicity ratios being obtained with SakSTAR (K74Q) and SakSTAR (K74R). SakSTAR (E80A,D82A) was preferred over the single residue variants SakSTAR (E80A) or SakSTAR (D82A) because of its somewhat lower reactivity with immunized patient plasma. SakSTAR (N95A) could not be further improved by substitution of N95 with [E, G, K or R] Glu, Gly, Lys or Arg and it was unable to confer its increased specific activity to variants containing K74A or K135R. Finally SakSTAR (K130A) was outperformed in terms of specific activity by SakSTAR (K130T) and SakSTAR (V132A) by SakSTAR (V132R).

On page 30, please delete and replace the current version of the second full paragraph starting at line 14 with the following replacement paragraph:

The SakSTAR (K130T,K135R) variant was taken as a template for additive mutagenesis because of its high specific activity with a moderate reduction of binding to antibodies of epitope cluster III and absorption of antibodies from immunized patient plasma (Table 6). Addition of G36R, K74R, or K74Q or both to the template did not markedly reduce the specific activity, reduced the reactivity with monoclonal antibodies against epitope cluster III (G36R substitution) and decreased the absorption of antibodies from immunized patient plasma (K74R or K74Q substitution). Combination of E65A or E65Q with K74Q in the SakSTAR (K130T,K135R) template reduced the absorption of antibodies from Pool 10 and Pool 40 to around 50 and 60 percent respectively, without markedly reducing the specific activity.

Addition substitution of selected amino acids in the SakSTAR (E65Q,K74Q,K130T,K135R) template did not further reduce the antibody absorption from Pool 10 or Pool 40. Surprisingly, the substitution of K136 with [A] Ala and the addition of [K] Lys in position 137 resulted in a marked increase in specific activity, as measured in the chromogenic substrate assay.

On pages 43 and 44, please delete and replace the current version of the first full paragraph starting at page 43, line 19 and bridging page 44 and ending at line 5 with the following replacement paragraph:

The variants SakSTAR (K102C) and SakSTAR (K109C), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using pMEX.SakSTAR encoding SakSTAR as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the staphylokinase gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC). The forward and backward primers shared an overlap of around 24 bp (for the construction of K102C: TAT GAT AAG AAT TGC AAA AAA GAA GAA (SEQ ID NO: 7) (backward) and TTC TTC TTT TTT GCA ATT CTT ATC ATA (SEQ ID NO: 8) (forward), for the construction of K109C: AAA AAG AAG AAA CGT GCT CTT TCC CTA (SEQ ID NO: 9) (backward) and TAG GGA AAG AGC ACG TTT CTT CTT TTT (SEQ ID NO: 10) (forward)). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of pMEX.SakSTAR. For each construction, the sequence of the variant was confirmed by sequencing the entire coding region.

On pages 49 and 50, please delete and replace the current version of the second full paragraph starting on page 49, line 31 and bridging page 50 and ending at line 21 with the following replacement paragraph:

The variants SakSTAR(S3C,E65D,K74R,E80A,D82A,K130T,K135R), (SY19(S3C)), SakSTAR(S2C,S3C,E65D,K74R,E80A,D82A,K130T,K135R), (SY19(2SC,3SC)), SakSTAR(S3C,K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T, K135R,K136A,∇137K), (SY141(S3C)), SakSTAR(S2C,S3C,K35A,E65Q,K74Q,D82A, S84A, T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K), (SY141(S2C,S3C)), SakSTAR(S3C,K35A,E65Q,K74Q,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T, K135R), (SY160(S3C)) and SakSTAR(S3C,K35A,E65Q,K74R,E80A,D82A,T90A,E99D, T101S,E108A,K109A,K130T,K135R), (SY161(S3C)), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using pMEX.SakSTAR encoding SakSTAR as template, two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the staphylokinase gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp. The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of pMEX.SakSTAR. For each construction, the sequence of the variant was confirmed by sequencing the entire SakSTAR coding region.

MARKED-UP VERSION OF THE CLAIMS

39. (Once Amended) [Staphylokinase derivatives] A staphylokinase derivative having essentially the amino acid sequence as depicted in figure 1 (SEQ ID NO: 1) [in which one or more amino acids have been replaced by another amino acid thus reducing the] reactivity with a panel of murine monoclonal antibodies specific towards staphylokinase and having in addition either one or both of the following:

(a) at least one amino acid substituted with Cys, [resulting in dimerization and/or increased specific activity and/or reduced clearance and/or increased thrombolytic potency] wherein the substitution is introduced at a position outside both the binding epitope and activation epitope of the staphylokinase molecule, and wherein the substitution allows the formation of a homodimeric form of staphylokinase through the formation of an intermolecular disulfide bridge; and/or

(b) polyethylene glycol [substitution] coupling to an amino acid residue, wherein the coupling is introduced at a position outside both the binding epitope and the activation epitope, resulting in a significantly reduced plasma clearance while maintaining specific activity.

40. (Once Amended) Staphylokinase derivatives as claimed in claim 39 having essentially the amino acid sequence as depicted in figure 1 (SEQ ID NO: 1) in which one or more amino acids have been replaced by another amino acid thus reducing the absorption of SakSTAR (a specific wild-type variant of staphylokinase)-specific antibodies from plasma of patients treated with staphylokinase.

41. (Once Amended) Staphylokinase derivatives as claimed in claim 39 having essentially the amino acid sequence as depicted in figure 1 (SEQ ID NO: 1) in which one or more amino acids have been replaced by other amino acids, [without reducing the specific activity by more than 50 percent] wherein the specific activity of said derivatives is at least 50% that of the corresponding wild-type staphylokinase.

43. (Once Amended) Staphylokinase derivatives as claimed in claim 39 and listed in Tables 1, 3, 4, 5, 6, 7, 8, 13, 19 and 20, having the amino acid sequence as depicted in figure 1 (SEQ ID NO: 1) [in which the indicated amino acids have been replaced by other amino acids] thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase, without reducing the specific activity.

45. (Once Amended) Staphylokinase derivatives as claimed in claim 39, wherein the Cys is chemically modified with polyethylene glycol [with molecular weights] wherein the polyethylene glycol can have a molecular weight of up to 20 kDa.

46. (Twice Amended) [Staphylokinase] The staphylokinase derivatives [as claimed in] of claim 45, wherein (a) selected amino acids in the NH_2-terminal region of 10 amino acids (SEQ ID NO: 1 positions 1-10), are substituted with Cys, which is chemically modified with polyethylene glycol and [is] (b) said derivatives are characterized by a significantly reduced plasma clearance and maintained thrombolytic potency upon single intravenous bolus administration at a reduced dose.

47. (Once Amended) Staphylokinase derivative as claimed in claim 46, wherein the [serine] Ser in position 2 or 3 (SEQ ID NO: 1) is substituted with a [cystein] Cys and the [cystein] Cys is chemically modified with polyethylene glycol having a molecular weight of 5, 10 or 20 kDa.

51. (Once Amended) [Staphylokinase] The staphylokinase derivative [as claimed in] of claim 47 labeled SY19 (S3C-MP5), wherein said [, which] derivative [is SakSTAR] comprises amino acid substitutions [( ] S3C-MP5, E65D, K74R, E80A, D82A, K130T, K135R [ ) having the code of SY19 (S3C-MP5)] and wherein the cysteine at position 3 is chemically modified with MAL-PEG 5 kDa.

APPENDIX A

39. (Once Amended) A staphylokinase derivative having essentially the amino acid sequence as depicted in figure 1 (SEQ ID NO: 1) reactivity with a panel of murine monoclonal antibodies specific towards staphylokinase and having in addition either one or both of the following:

(a) at least one amino acid substituted with Cys, wherein the substitution is introduced at a position outside both the binding epitope and activation epitope of the staphylokinase molecule, and wherein the substitution allows the formation of a homodimeric form of staphylokinase through the formation of an intermolecular disulfide bridge; and/or

(b) polyethylene glycol coupling to an amino acid residue, wherein the coupling is introduced at a position outside both the binding epitope and the activation epitope, resulting in a significantly reduced plasma clearance while maintaining specific activity.

40. (Once Amended) Staphylokinase derivatives as claimed in claim 39 having essentially the amino acid sequence as depicted in figure 1 (SEQ ID NO: 1) in which one or more amino acids have been replaced by another amino acid thus reducing the absorption of SakSTAR (a specific wild-type variant of staphylokinase)-specific antibodies from plasma of patients treated with staphylokinase.

41. (Once Amended) Staphylokinase derivatives as claimed in claim 39 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by other amino acids, wherein the specific activity of said derivatives is at least 50% that of the corresponding wild-type staphylokinase.

43. (Once Amended) Staphylokinase derivatives as claimed in claim 39 and listed in Tables 1, 3, 4, 5, 6, 7, 8, 13, 19 and 20, having the amino acid sequence as depicted in figure 1 (SEQ ID NO: 1) thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase, without reducing the specific activity.

45. (Once Amended) Staphylokinase derivatives as claimed in claim 39, wherein the Cys is chemically modified with polyethylene glycol wherein the polyethylene glycol can have a molecular weight of up to 20 kDa.

46. (Twice Amended) The staphylokinase derivatives of claim 45, wherein (a) selected amino acids in the NH_2-terminal region of 10 amino acids (SEQ ID NO: 1 positions 1-10), are substituted with Cys, which is chemically modified with polyethylene glycol and (b) said derivatives are characterized by a significantly reduced plasma clearance and maintained thrombolytic potency upon single intravenous bolus administration at a reduced dose.

47. (Once Amended) Staphylokinase derivative as claimed in claim 46, wherein the Ser in position 2 or 3 (SEQ ID NO: 1) is substituted with a Cys and the Cys is chemically modified with polyethylene glycol having a molecular weight of 5, 10 or 20 kDa.

51. (Once Amended) The staphylokinase derivative of claim 47 labeled SY19 (S3C-MP5), wherein said derivative comprises amino acid substitutions S3C-MP5, E65D, K74R, E80A, D82A, K130T, K135R and wherein the cysteine at position 3 is chemically modified with MAL-PEG 5 kDa.

61. The staphylokinase derivative of claim 39, wherein the at least one amino acid substituted with Cys is at least one of a surface exposed residue, a charged residue, a threonine residue and a serine residue.

62. The staphylokinase derivative of claim 39, wherein the at least one amino acid substituted with Cys is the position of the polyethylene glycol coupling.

IDENTIFICATION, PRODUCTION AND USE OF STAPHYLOKINASE DERIVATIVES WITH REDUCED IMMUNOGENICITY AND/OR REDUCED CLEARANCE

ABSTRACT OF THE DISCLOSURE

Methods for the identification, production and use of staphylokinase derivatives characterized by a reduced immunogenicity after administration in patients and that can be administered by a single bolus injection. The derivatives of the invention are obtained by preparing a DNA fragment comprising at least part of the coding sequence of staphylokinase; performing *in vitro* site-directed mutagenesis on the DNA fragment; cloning the mutated DNA fragment in a suitable vector; transforming a suitable host cell with the vector; culturing the host cell under conditions suitable for expressing the DNA fragment; purifying the staphylokinase derivative and chemically modifying Cys residues with thiol-directed polyethylene glycol. The invention also relates to pharmaceutical compositions comprising at least one of the staphylokinase derivatives according to the invention together with a suitable excipient, for treatment of arterial thrombosis.